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POSSIBLE USE OF *BACILLUS THURINGIENSIS* BERLINER AS AN AID IN THE BIOLOGICAL CONTROL OF THE ALFALFA CATERPILLAR[1]

EDWARD A. STEINHAUS[2]

INTRODUCTION

INASMUCH AS BACTERIA that form endospores might be expected to be more useful as biological control agents than those that do not form endospores, it was decided to make a preliminary test of the pathogenicity of available sporeformers for certain lepidopterous pests in California. Among the bacteria tested was the sporeformer *Bacillus thuringiensis* Berliner, which was found to be particularly virulent for the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval, although larvae of certain other species were also susceptible to the bacterium. On the basis of the marked susceptibility of the alfalfa caterpillar to infection by the microorganism in the laboratory, preliminary tests were made in the field to ascertain any potential value *B. thuringiensis* might have as a control agent. The present paper is a report of the results obtained in these tests. Also presented are certain aspects of the biological control potentialities of other sporeforming bacteria, and some general considerations of *B. thuringiensis* as an insect pathogen.

SOURCE AND IDENTITY OF BACILLUS

In 1911 Berliner,[3] in Germany, published a preliminary report on a disease of the larva of the Mediterranean flour moth, *Ephestia kühniella* Zeller, caused by a sporeforming bacterium which he later (1915) named *Bacillus thuringiensis*. The disease first appeared in a shipment of larvae received by Berliner in the summer of 1909 from a flour mill in Thuringia, Germany. It soon spread to other *E. kühniella* larvae throughout the "Experiment Station for Grain Processing," enabling the German worker to study the disease for several consecutive years. Berliner found the bacillus to be pathogenic primarily for larvae of the Mediterranean flour moth, while showing no infectivity for larvae of other Lepidoptera (e.g., certain species of *Nygmia*,

[1] Contribution from the Laboratory of Insect Pathology, Division of Biological Control, College of Agriculture, University of California, Berkeley. Manuscript submitted for publication October 28, 1950.

[2] Associate Professor of Insect Pathology and Associate Insect Pathologist in the Experiment Station, Berkeley.

[3] See "Literature Cited" for citations, referred to in the text by author and date.

Lymantria, and *Thaumetopoea*), and larvae and adults of certain Coleoptera (e.g., *Calandra, Tribolium, Dermestes,* and *Tenebrio*).

The strain of *Bacillus thuringiensis* (figs. 1, 2, 3, 4, 10) used in the experiments herein reported was kindly furnished the author in 1942 by Nathan R. Smith who had received it from J. R. Porter in 1940. Porter had previously, in 1936, obtained the organism directly from Otto Mattes in Germany. It is apparently the same strain isolated from diseased larvae of the Mediterranean flour moth by Mattes and reported by him in 1927. Although Mattes spelled the name *"Bacillus thuringensis,"* the organism is apparently the same as that described by Berliner; Mattes' omission of the letter "i" undoubtedly was unintentional. According to Smith, Gordon, and Clark (1946) the strain of *B. thuringiensis* they studied, as well as the descriptions of this species by Berliner and by Mattes, correspond to the well-known *Bacillus cereus* Frankland and Frankland, commonly found in soil. A description of *B. thuringiensis* by Chorine (1929) also is in accord with that of *B. cereus* except that Chorine reports the fermentation of mannitol, which is not characteristic of *B. cereus* or of the strain of *B. thuringiensis* we obtained from Dr. Smith. Metalnikov and Chorine (1929*c*) isolated from diseased larvae of the Mediterranean flour moth a sporeforming bacterium they named *Bacterium ephestiae.* Later, Ellinger and Chorine (1930) found this organism to be the same as *B. thuringiensis,* making it also a strain of *B. cereus.*

Other Entomogenous Strains of *B. cereus*

Another sporeforming bacterium reported to be pathogenic for certain insects and now known to be a strain of *B. cereus* is that studied by Sokoloff and Klotz (1942) and designated by them as *Bacillus* "C." However, their report concerning the invasive properties of this strain for the California red scale has not been confirmed (see Steinhaus, 1949). *Bacillus mycoides* Flügge, now considered a variety of *B. cereus,* has been found to be experimentally pathogenic for the silkworm, *Bombyx mori* (Linn.), and the larva of the wax moth, *Galleria mellonella* (Linn.). Also possibly synonymous with *B. cereus* is *Bacillus hoplosternus* Paillot, isolated by Paillot (1919) from diseased cockchafers, *Melolontha melolontha* (Linn.), and found to be pathogenic for several lepidopterous larvae (*Nygmia, Malacosoma, Arctia,* and *Vanessa.*) The gypsy-moth caterpillar, *Porthetria dispar* (Linn.), appeared to be immune to the bacillus. *Bacillus ellenbachi* Sawamura (*Bacillus ellenbachensis* Gottheil?), referred to by Sawamura (1906) as producing flacherie in silkworms, may also be *B. cereus,* as is *Bacillus albolactis* (Loeffler) Migula, recovered by Hatcher (1939) from the oral cavity and feces of the American cockroach, *Periplaneta americana* (Linn.).

A bacterium recognized as *Bacillus cereus* has been observed by Babers (1938) as the cause of a septicemia in the southern armyworm, *Prodenia eridania* Dram. This strain was also capable of infecting the American cockroach when injected into its body cavity. Strains of *B. cereus* identified by N. R. Smith as equivalent to *B. thuringiensis* have been isolated by the present author from diseased larvae of the Indian mealworm, *Plodia interpunctella* (Hbn.), and *Aphomia gularis* (Zeller). Typical strains of *B. cereus* have also been isolated from apparently healthy insects and ticks (Steinhaus, 1946*b*).

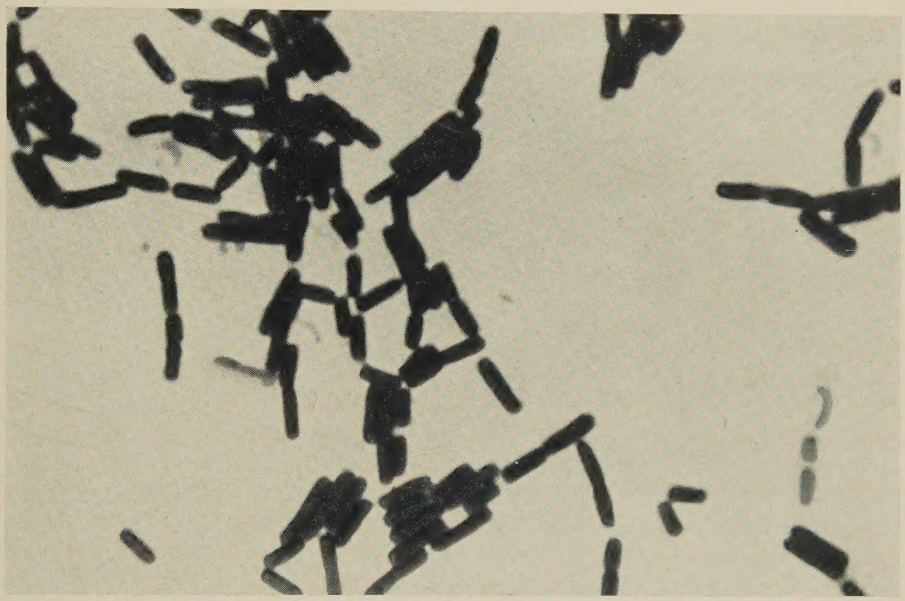

Fig. 1. Vegetative cells of *Bacillus thuringiensis* Berliner from a 20-hour culture on nutrient agar. Magnification approximately 2000 ×.

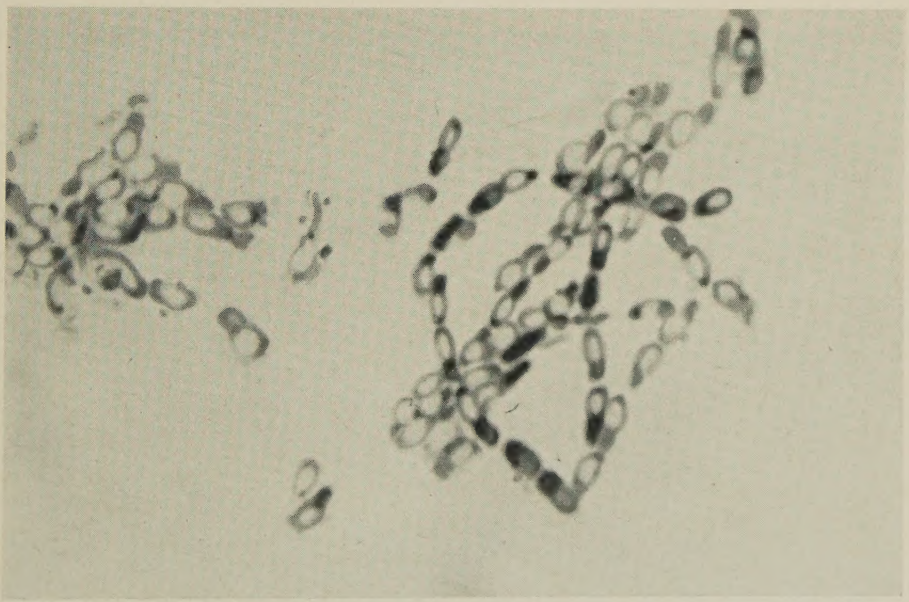

Fig. 2. Spores of *Bacillus thuringiensis* from a 32-hour culture on nutrient agar. Spores still contained within the bacterial cells (sporangia). An occasional sporangium with obliquely lying spore may be seen, although this phenomenon is not well shown in this particular preparation. Magnification approximately 2500 ×.

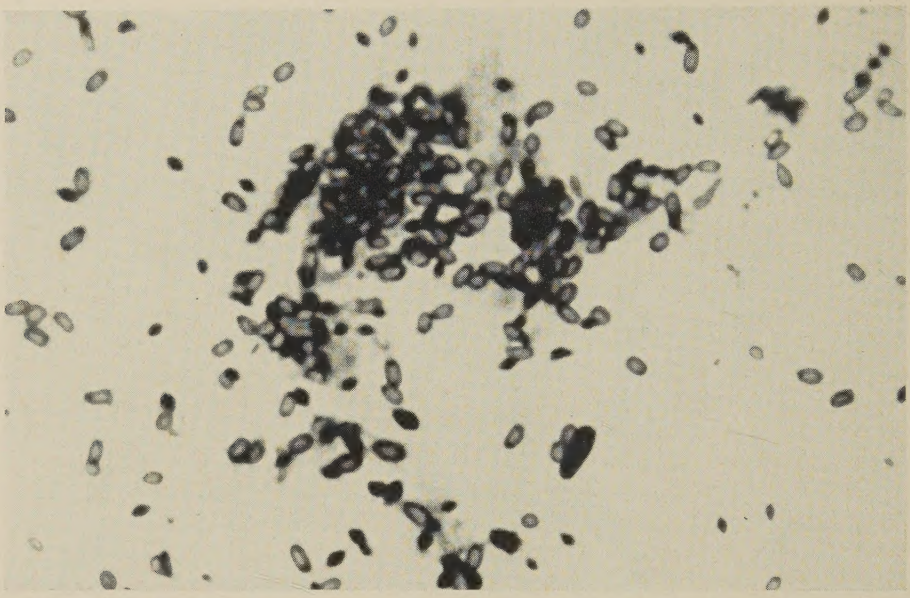

Fig. 3. Spores of *Bacillus thuringiensis* from a three-day culture on nutrient agar. Most of the spores have become free of the sporangia or remains of the vegetative cells. Magnification approximately 2000 ×.

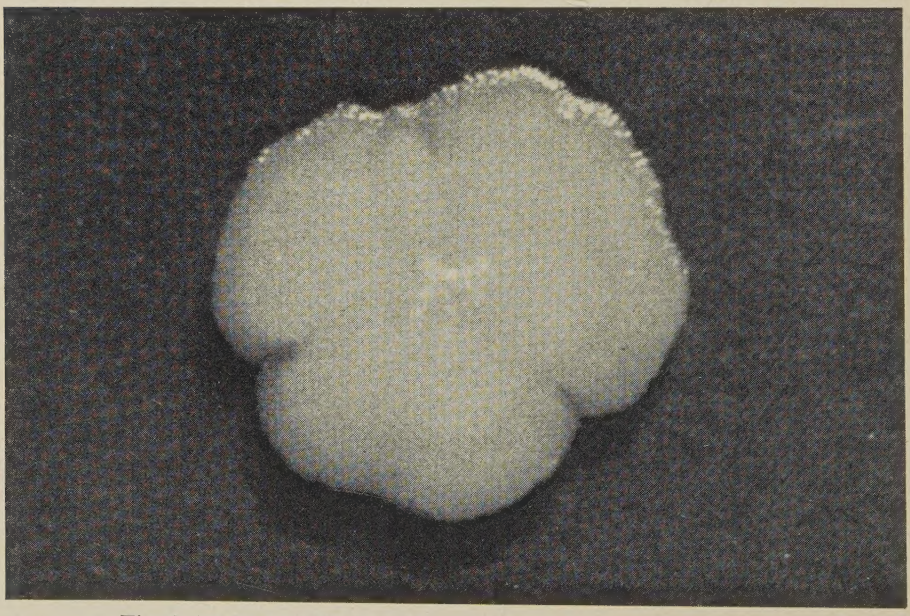

Fig. 4. A colony of *Bacillus thuringiensis* growing on nutrient agar. Magnification approximately 5.4 ×.

COMPARATIVE PATHOGENICITY AND NOMENCLATURE

In light of the finding by Smith, Gordon, and Clark that the Mattes strain of *B. thuringiensis* corresponded morphologically and culturally to *B. cereus*, it was thought wise to determine the possible pathogenicity of several strains of typical *B. cereus* isolated from the soil and other sources and to compare these with that of *B. thuringiensis*. If *B. cereus* proved to have the same capabilities to infect certain insects as *B. thuringiensis* is known to have, there would be little reason to continue distinguishing one from the other. Accordingly comparative infectivity tests were set up.

Nine strains of *B. cereus* (seven from N. R. Smith, USDA strains 201, 205, 232, 244, 249, 701, 793) originally isolated from various sources including three from soil, three from diseased small vertebrates, and three from an old stock culture were used in this test. Forty-eight-hour cultures, grown on nutrient agar and then washed off to make an aqueous suspension, were fed[4] to the test insects; the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval; the western yellow-striped armyworm, *Prodenia praefica* Grote; the California oakworm, *Phryganidia californica* Packard; the variegated cutworm, *Peridroma margaritosa* (Haworth); and the buckeye caterpillar, *Junonia coenia* Hbn. The infectious feeding was accomplished by dipping the foliage used as the insect's food in a concentrated suspension of each test bacillus just prior to giving it to the insect. For each strain of *B. cereus* six larvae of each species of insect were used, except in the case of the alfalfa caterpillar where ten larvae were used to test each strain of bacillus.

None of the nine strains of *B. cereus* showed any consistent degree of pathogenicity for any of the five test insects. Four of the strains (232, 244, 249, 793) each killed one out of ten alfalfa caterpillars used in the test. In each of these instances the sporeformer was found in the body contents of the dead caterpillar. However, it is felt that this did not represent any significant indication of pathogenicity, especially when compared with *B. thuringiensis* which at the same time killed ten out of ten caterpillars. Additional tests with *B. thuringiensis* under the same conditions as those prevailing in these tests showed a mortality rate of practically 99 per cent. The pathogenicity of *B. thuringiensis* for the other species of test insects, as determined in the laboratory, was somewhat less than for the alfalfa caterpillar. *Peridroma* larvae appeared to be the least susceptible, with the larvae of *Junonia, Phryganidia, Prodenia,* showing moderate degrees of susceptibility.

It appears that *B. thuringiensis,* although culturally and morphologically (except as noted in footnote, p. 366) similar to *B. cereus,* has the property of being pathogenic for insects while most strains of typical *B. cereus* are not. This fact in itself would not warrant considering *B. thuringiensis* a species

[4] In our experience the pathogenicity of most bacteria for most insects can usually best be determined by feeding the bacterium to the insect directly or including it along with the insect's food. Direct inoculation of the bacteria into the body cavity of the insect is an inadequate test since many common saprophytes, having little or no invasive power, find the hemolymph a satisfactory medium and one in which they develop well, thereby killing the host as a result of their luxuriant growth. Such mortality, however, does not necessarily represent true pathogenicity for the insect, and does not indicate the natural susceptibility of the host to the microorganisms.

distinct from *B. cereus*. However, the pathogenicity of *B. thuringiensis* for insects does seem to be a differential characteristic which is persistent, presenting a situation similar to that existing between *B. cereus,* usually saprophytic, and *Bacillus anthracis* Cohn *emend.* Koch, the causative agent of anthrax. For this reason, and because of the possible economic significance involved, it would seem that for practical purposes the name *Bacillus thuringiensis* should be retained for the present and kept separate from *Bacillus cereus*. This arrangement would also allow for a continuity of nomenclature in entomological literature where the designation *B. thuringiensis* has been used since Berliner proposed it in 1915. In this paper, therefore, we shall consider *B. thuringiensis* as a species closely related taxonomically to *B. cereus* and *B. anthracis,* differentiated primarily by being pathogenic for certain insects. If eventually it can be shown that strains of *B. cereus* may be derived from *B. thuringiensis,* or vice versa, it would probably then be wise to assign *B. thuringiensis* to a subspecific category. There is need for further exploration of the whole problem of comparative pathogenicity and its inheritance and variability.

Symptoms

Susceptible larvae of most species of Lepidoptera show similar symptoms following infection by *Bacillus thuringiensis*. In the case of the alfalfa caterpillar, the insect of primary importance in this report, the first detectable signs of infection are the apparent loss of appetite and the sluggish mobility of the insect, occurring within a few hours after ingestion of the bacillus. Also observable is a decreased response to tactile stimulation. As the disease progresses there is some diarrhea, as evidenced by the tendency of the larva to adhere to the walls of the rearing cage at a point where its anus is "stuck" to the surface. The bacillus eventually invades the body cavity of the insect, causing a septicemia. The first signs of discoloration may be a darkened area or spot on the caterpillar's back or, less frequently, on one side or the other. As death approaches the insect may become somewhat shrunken in appearance, or it may be stretched out in an entirely flaccid condition. The latter is more common; in either case the larva is frequently found lying on its side and is unable to remain upright when righted.

Shortly after the caterpillar dies its entire body may become more or less discolored, assuming a dark olive-green to dark brown or black color. Such discoloration does not always occur, however, and its intensity may be dependent upon the activity of secondary invaders or saprophytes. The soft, flaccid, collapsed cadaver has little or no tendency to resume its normal shape after its body has been depressed with a blunt instrument. The body contents of the dead insect may be soft and somewhat fluid (fig. 5). Occasionally there is some disintegration of the integument, but in most instances the integument remains intact. The dead larvae usually give off a putrefying odor resembling that of "spoiled alfalfa."

Although the pathogenesis or course of infection as it takes place in the insect host has not been studied in detail or correlated with the symptomatology of the disease in the alfalfa caterpillar, it has been described by Mattes (1927) as it pertains to the infection in the Mediterranean flour moth, *Ephestia kühniella* Zeller. He depicts the following successive stages: (*1*)

Ingestion of the spores and their germination in the midgut of the insect; (*2*) formation of large numbers of vegetative cells in the gut; (*3*) damaging the gut epithelium by the enzymatic activity of the bacillus as it flourishes in the gut contents; (*4*) migration of the bacteria between the host's cells into the body cavity (according to Berliner, 1915, the bacteria enter the body cavity through openings in the gut wall that result from the disintegrating

Fig. 5. Larvae of the alfalfa caterpillar dead of infection with *Bacillus thuringiensis*. From a sample of specimens collected in the field following the spray application of the spores of the bacillus.

process); (*5*) abundant growth of the bacillus in the hemolymph; (*6*) disintegration of internal tissues (e.g., the fat body) as the result of bacterial enzymes; (*7*) appearance of the external symptoms of the disease; (*8*) penetration of the bacteria into the tissues of the nervous system, resulting in the death of the larva; (*9*) further disintegration of all tissues and the drying or "mummification" of the larval remains; (*10*) formation of spores by the bacteria.

It might be mentioned here that *B. thuringiensis* showed no pathogenicity for rabbits when inoculated intradermally, subcutaneously, intraperitoneally, or perorally. Oral consumption of a culture of the bacillus by a human volunteer produced no untoward results of any kind. Berliner (1915) also found no indication that the bacillus was in any sense pathogenic for vertebrates.

Pathogenicity of Other Sporeformers

Incident to the studies being reported here, 37 additional strains of sporeformers of various species were tested for their capabilities of infecting the larvae of the five insect species listed above. Most of these strains in the following list were obtained from Dr. N. R. Smith; the figures in parentheses are his numbers.

B. megatherium De Bary (N. R. Smith's strains nos. 234, 239)
B. cereus var. *mycoides* (Flügge) (233, 936)
Bacillus "C" Sokoloff and Klotz (*B. cereus* Frankland and Frankland)
B. subtilis Cohn *emend.* Prazmowski (231, 243)
B. subtilis var. *aterrimus* (Lehmann and Neumann) Smith, Gordon, and Clark (653)
B. subtilis var. *niger* (Migula) Smith, Gordon, and Clark (220)
B. pumilus Gottheil (236, 272)
B. coagulans Hammer (609, 770)
B. firmus Werner (613, 1153)
B. lentus Gibson (670)
B. pasteurii (Miquel) Chester (929)
B. polymyxa (Prazmowski) Migula (279)
B. macerans Schardinger (278, 1098)
B. circulans Jordan (358, 746)
B. krzemieniewski Kleczkowska, Norman, and Snieszko (*B. circulans* var.?) (760)
B. alvei Cheshire and Cheyne (662, 686)
B. laterosporus Laubach (314, 661)
B. brevis Migula *emend.* Ford (604, 616)
B. sphaericus Neide (344, 732)
B. sphaericus var. *rotans* (Roberts) Smith, Gordon, and Clark (633)
B. sphaericus var. *fusiformis* (Gottheil) Smith, Gordon, and Clark (339, 592)
Bacillus isolated from *Prodenia praefica* Grote
Bacillus isolated from *Aphomia gularis* (Zeller)
Bacillus isolated from *Peridroma margaritosa* (Haworth)

Cultures of nearly all of these strains had been maintained on artificial media for considerable periods of time since their original isolation and prior to their being used in this test. Probably none, however, had undergone artificially maintained cultivation much longer than had *B. thuringiensis*.

Each of the 37 strains were fed to at least ten larvae of each of the test insect species (except in some cases where *Prodenia praefica* Grote was not used because of its unavailability); in a few instances 20 test insects were used. Only one of the 37 strains exhibited any significant degree of pathogenicity for the test larvae, it being only slightly less pathogenic than the Mattes strain of *B. thuringiensis*. The bacillus concerned in this instance was one isolated in our laboratory from a diseased larva of the moth *Aphomia gularis* (Zeller) found in a local almond-shell bin. It has been identified by N. R. Smith as a strain of *Bacillus thuringiensis*.[5] *Junonia, Prodenia,* and

[5] In 1945, the author isolated from diseased larvae of the Indian mealworm, *Plodia interpunctella* (Hbn.), a sporeforming bacillus which was originally identified by Dr. N. R. Smith as a strain of *Bacillus cereus*. Recently Smith had occasion to compare this strain with our strain from *Aphomia* and the Mattes strain of *B. thuringiensis*. In the case of all three strains, a rather peculiar sporangium was observed. The spore lies obliquely which results in two knobs of protoplasm being left at each end, usually more at one end than the other. When the sporangium disintegrates these knobs remain intact giving somewhat the appearance of cocci. In Smith's opinion, all three strains, although from different insect

Colias larvae were all rather highly susceptible to the organism, *Phryganidia* was less so and *Peridroma* only slightly if at all. Virtually no pathogenicity for the test insects was shown by any of the remaining 36 strains. It is possible that some virulence for the insect might have been developed in certain cases by passing the bacteria through the insect host several times via direct inoculation prior to feeding them. On the basis of other experiences along this line, however, it is doubtful whether this would occur in the majority of cases, and it is fairly certain that the newly acquired pathogenicity would not be as permanent or long-lasting as it is with *B. thuringiensis*.

Known true and adequately described insect pathogens among the sporulating bacteria are relatively few in number. These include: *Bacillus alvei* Cheshire and Cheyne, *Bacillus larvae* White, *Bacillus laterosporus* Laubach (=*Bacillus orpheus* White), and *Bacillus pulvifaciens* Katznelson, all pathogenic for the honeybee; *Bacillus lentimorbus* Dutky and *Bacillus popilliae* Dutky, pathogens of the Japanese beetle; and *Bacillus bombycis* auctt., infectious for the silkworm (see Steinhaus, 1946*a*). Whether or not *B. thuringiensis* warrants a place in this list will depend on the outcome of studies concerning its basic relation to *B. cereus* and the possible variability of its pathogenicity for insects.

PRELIMINARY FIELD TESTS

On the basis of the distinct virulence shown in the laboratory by *Bacillus thuringiensis* for the alfalfa caterpillar, it was decided to conduct preliminary field tests to determine the susceptibility of the insect to the bacterium as it might occur under field conditions. To our knowledge this bacillus had never been tried as a biological control agent against the alfalfa caterpillar or, for that matter, against any insect infesting a crop of the same type as alfalfa. Furthermore, the experiments herein reported represent the first time that *B. thuringiensis* has been studied to any extent as a possible biological control agent in the United States, although White (1927) in Washington, D.C., did note its presence in laboratory cultures of *Ephestia*.

In his 1915 paper, Berliner treats briefly the possible practical use that could be made of *B. thuringiensis* in suppressing infestations of the Mediterranean flour moth in granaries and flour mills. Although his few experiments indicated that no great success attended the spraying or dusting of the machinery and walls of a mill, Berliner did feel that the actual incorporation of spores with the grain and mill products could be an effective means of controlling the pest. Similarly Shepherd, in 1924, mentions the use of the bacillus as a means of controlling a cosmopolitan bran beetle, *Echocerus cornutus* (Fabr.), in Germany. Husz (1928) (as apparently did Prell in unpublished

sources, are the same. However, *Bacillus* "C" of Sokoloff and Klotz (which has been identified as a strain of *Bacillus cereus* but for which the pathogenicity for insects has not been confirmed) does not show the same peculiar sporangium. On the basis of these few observations Smith is not willing to conclude definitely that virulence is indicated by the oblique spores, but considers that it might be. In our own laboratory we have noticed one interesting difference between these various strains. Whereas the Mattes strain of *Bacillus thuringiensis* and *Bacillus* "C" gave a positive egg-yolk reaction (McGaughey and Chu, 1948), the strains from *Plodia* and *Aphomia* were negative in this respect.

experiments) showed the European corn borer, *Pyrausta nubilalis* (Hbn.), to be susceptible to *B. thuringiensis*, and in 1929, 1930, and 1931 reported favorable results in using spore dusts and sprays in combating this insect. Although they did not make actual field tests, Metalnikov and Chorine (1929*a*, *b*, *c*) also found the organism to be highly virulent for the European corn borer, as well as for larvae of *Porthetria dispar* (Linn.), *Aporia crataegi* Linn., and *Vanessa urticae* (Linn.), but not for certain grasshoppers, mosquitoes, and beetles. Similar reports concerning the susceptibility of the corn borer both in the laboratory and in the field have been made by Chorine (1930*a*, *b;* 1931), Metalnikov, Ermolaev, and Skobaltzyn (1930), and Metalnikov, Hergula, and Strail (1930*a*, *b;* 1931).

Preparation of Spore Material

The fact that *B. thuringiensis* grows well on ordinary nutrient agar without loss of virulence greatly facilitates its production in appreciable quantities. The spore material used in the field tests about to be described was grown on nutrient agar placed in six-liter Povitsky bottles. The offset neck of each bottle is such that the medium is safely contained when the bottle is placed on its side (Fig. 6). The solidified agar thus presents a surface of approximately 75 square inches for growing the bacteria. Cursory, preliminary tests indicated that the addition of 1 per cent dextrose to the nutrient agar medium did not appreciably increase the number of spores produced on the medium over that produced on plain nutrient agar alone. The total number of spores formed in a liquid medium (nutrient broth) was considerably less than that obtained from a solid medium (nutrient agar). For these reasons all the spores used in the field experiments were produced on plain nutrient agar. It was found, however, that the addition of 1 per cent dextrose to nutrient agar frequently did increase the *rate* of spore formation, particularly during the first 24 hours. This finding was not consistent, however, and within 48 hours spore formation on plain nutrient agar was almost equivalent to that on nutrient agar to which dextrose had been added. Sporulation on both types of media was usually complete within 72 hours. The addition of 2 per cent proteose-peptone or 5 per cent sodium chloride to the nutrient agar appeared to retard the rate of spore formation.

The medium in each bottle was inoculated by spraying the surface of the agar with a 24- to 48-hour diluted culture of the bacillus. A small hand atomizer used for this purpose ensured an even distribution of the inoculum. The bottles were incubated at room temperature (about 23° C) for varying lengths of time, usually about seven days. This average incubation period was selected on the basis of Husz's (1929) statement that six days after inoculation the culture consists entirely of spores. In our own laboratory we found spore formation on nutrient agar to be almost complete after two or three days. It appears likely, therefore, that very little is gained by prolonging the incubation period beyond three days, although this may be necessary under different conditions or when other media are used.

Upon completion of the incubation period, the spores are harvested by washing the growth off the agar with about 100 ml of sterile distilled water. The resulting suspension is washed once by centrifugation, filtered through

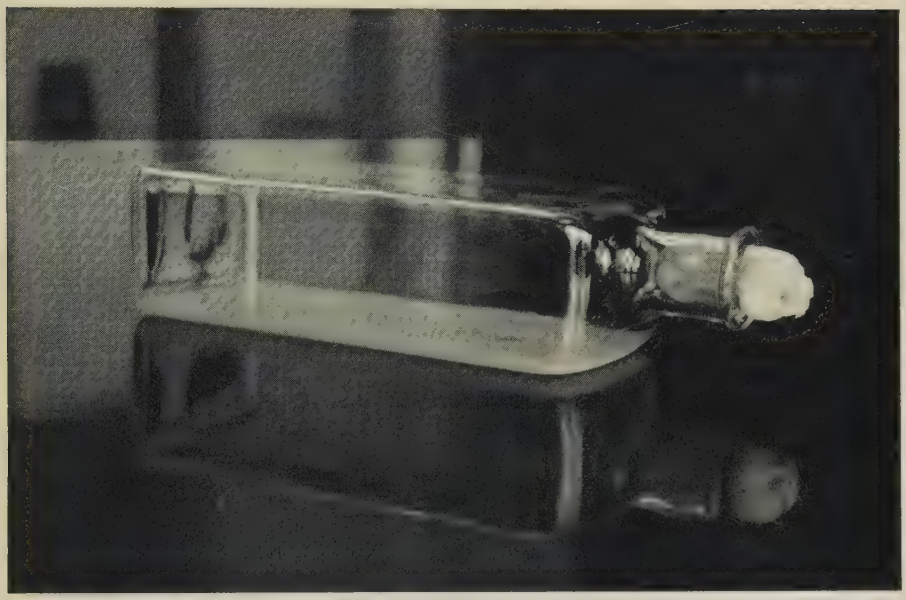

Fig. 6. Povitsky bottle used in the production of spores of *Bacillus thuringiensis*. The bottle contains solidified nutrient agar supporting the growth of the bacillus.

Fig. 7. Spore material after drying in pan and about to be scraped off as powder.

Fig. 8. Spore powder scraped from pan in fig. 7.

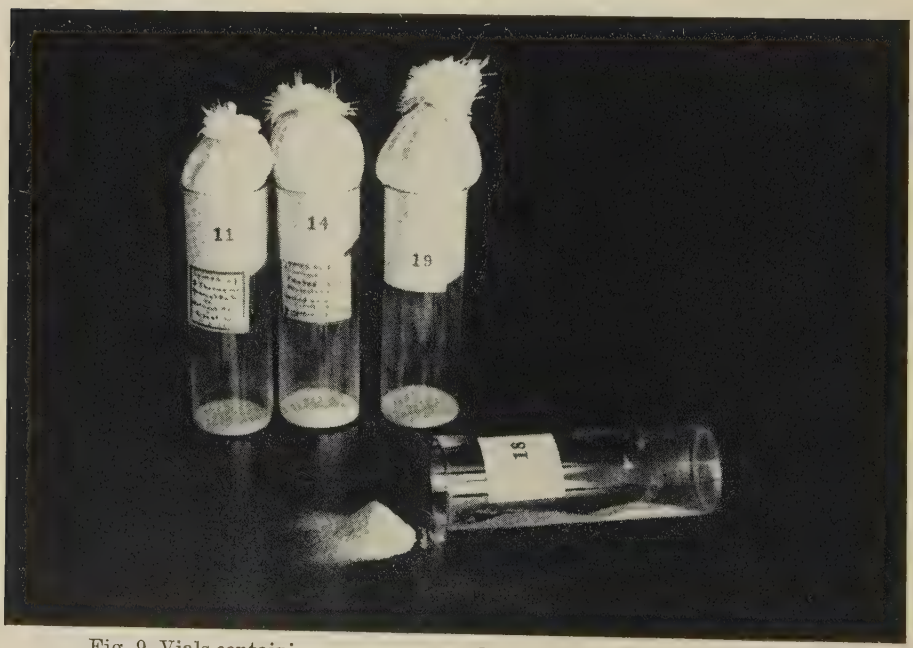

Fig. 9. Vials containing pure spore powder and in which experimental lots of spores were temporarily stored.

cheesecloth to remove stray bits of agar, and poured out onto flat enameled pans approximately 12 by 24 inches in size, and allowed to dry (fig. 7). At ordinary room temperatures evaporation of the water is usually completed in a 24-hour period. Commonly, however, the material is allowed to dry for 48 hours or longer. The dry, brittle deposit of spores is then scraped from the pan and triturated into a fine powder by grinding with a mortar and pestle (fig. 8). Each lot of dried spore powder is placed in a large glass vial and held in a dry place at room temperature until needed (fig. 9). Experiments still in progress indicate that dried spores retain their viability and virulence when stored in this manner for a period of at least a year, and probably considerably longer. Mattes (1927) found the spores to remain viable for at least six years in dry storage. The yield from 12 Povitsky flasks usually averaged between two and three grams of dried spore powder, or about 0.2 to 0.3 grams per flask.

Application of Spore Material

For the purpose of the experiments described in this report, field distribution of the bacteria was undertaken by very simple means. The concentrated spore powder was taken directly to the field where it was placed in suspension in an ordinary five-gallon back-pack hand sprayer, and then diluted with water to make the final spray solution. The amount of spore powder used on each test plot had been previously weighed in the laboratory so that the final concentration of spores could be determined by controlling the amount of water used as diluent. After thorough agitation in the sprayer, the suspension was applied by directing the spray uniformly over the alfalfa. In the case of the nine test plots covered in this report approximately two gallons of spray were applied to each plot except plot 9 for which almost four gallons were used.

Although a hand spray was used during the present investigation, there is probably no practical reason why effective field distribution of the infectious material could not be accomplished by the use of aircraft. Furthermore, it would seem entirely feasible to use spore dusts instead of sprays for the field applications. Laboratory experiments indicate that spore dusts give effective results in infecting alfalfa caterpillars feeding on their host plant.

All test plots were established in large fields of alfalfa on the Redfern Ranch near Dos Palos, California. Two series of test plots were set up, each in a separate field. The first series consisted of six test plots and three accompanying control plots, and ran from August 1 through August 4, 1950, with counts being made on the day of application and on the second and third days following. The second series consisted of three test plots and three accompanying control plots, and ran from August 29 through September 1, 1950, with counts being made at intervals of 0, 34, 58, and 82 hours. All plots in Series I were approximately 12 by 48 yards in size. The alfalfa had grown to about one-half its usual cutting height. At the time the bacterium was applied the field was under irrigation and the wind had a velocity of about 10 miles per hour. Approximately the same conditions prevailed at the time Series II was initiated. Test plots 7 and 8 were 15 by 48 yards in size, and the dimensions of plot 9 were 30 by 48 yards. The sizes of the control plots were the same as those of the corresponding test plots.

Inasmuch as these tests were of a preliminary nature only, and since their

primary purpose was to determine if the alfalfa caterpillar were susceptible to *B. thuringiensis* under field conditions, as it was under laboratory conditions, no effort was made to make the tests quantitative in nature. The approximate total concentration of spores (or clumps of spores) applied to each of the plots varied only slightly, when considered from a practical standpoint, as follows:

Series I	Series II
Plot 1 = 7,000,000,000	Plot 7 = 6,000,000,000
Plot 2 = 9,000,000,000	Plot 8 = 13,000,000,000
Plot 3 = 13,000,000,000	Plot 9 = 8,000,000,000
Plot 4 = 10,000,000,000	
Plot 5 = 13,000,000,000	
Plot 6 = 11,000,000,000	

To determine the rate at which the spores were applied (i.e., the number of spores per ml) these figures should be divided by 7517 (the number of mls of water used as diluent in making the spore suspension) for all plots but plot 9, in which instance the divisor should be 15,034.

The figures listed above are based on a determination of the average plate count of viable spores in four different lots of spores, assuming that the method of spore production used in preparing the various lots gives fairly uniform yields. Actual counts of the number of bacteria in each spore suspension as used in the field were not made. Furthermore, the figures listed represent clumps of spores as well as individual spores, inasmuch as the degree of agitation in the sprayer after the dried spore material was introduced into the water was not sufficient to break up the myriad small clumps of spores which naturally cohere under such circumstances. That such clumping actually occurs was revealed by simultaneously counting the colonies on nutrient agar plates arising from spores as they occur in an ordinary suspension and as they occur in a suspension vigorously shaken (about 50 times) with glass beads. Subsequent counts revealed that for identical lots of spore-material the average count was approximately 10 times as high in the case of the suspensions shaken with beads as in the suspensions not shaken. It is obvious that future experiments could benefit from a closer and more careful supervision of the quantitative aspects of the dosage used in field tests.

The age of the spores at the time of application (i.e., the period from the time the spores were produced on artificial media, harvested, and placed in dry storage, until they were applied in the field) varied with each plot as follows: plot 1, 9½ months; plot 2, seven months; plot 3, 4¾ months; plot 4, 3½ months; plot 5, 1½ months; plot 6, one month; plot 7, 6½ months; plot 8, 10¼ months; and plot 9, 12 months. These figures are cited for their informative value only. None of the field experiments was set up for the purpose of determining the influence that the age of the spores might have on the pathogenicity of the bacillus or its effectiveness in the field.

Results of Field Tests

The primary objective in the field tests was merely to determine if the alfalfa caterpillar could be infected and killed as it occurred in the field by

the artificial distribution of *B. thuringiensis*. That such can be accomplished was definitely proved. Within 24 to 48 hours following the spray applications, microscopic and cultural examinations of the dead and dying larvae showed the progress of the infection to be a vigorous one with large numbers of bacilli present in the body fluids and tissues of the insects (fig. 10). Diseased larvae showed typical symptoms and exhibited essentially the same post mortem appearances as those infected in the laboratory and described in an earlier paragraph of this paper.

Fig. 10. *Bacillus thuringiensis* as it appears in the fluids and tissues of the alfalfa caterpillar dying of infection by the bacillus. Magnification approximately 2000 ×.

In at least seven of the nine test plots the caterpillar population was brought to below an economic level (generally considered to be about 20 per two sweeps; Smith, 1949) whereas in each of the untreated control plots the population remained at a destructive level. In the remaining two of the nine test plots, the count was brought down to just slightly above 20, and would very probably have been reduced still further had the plots been allowed to remain a day or two longer. Unfortunately, all plots were cut for hay by the growers before the tests could be run to their desired completion.

The data for the nine test (treated) plots, as well as those for the accompanying control (untreated) plots are given in tables 1 and 2, and in figures 11 and 12.

The curves in figure 11 represent the average counts obtained in test plots 1–6, and in the three control plots, over a three-day period. The spore-spray was applied at a time when the count in the test plots averaged 44 larvae per two sweeps of the net. (The two-sweep counts were actually an average of five separate counts of two sweeps each.) At the end of a three-day period the count in the test plots had dropped to an average of 11 larvae per two sweeps

(well below the economic level), while that in the untreated control plots had risen to an average of 86 larvae per two sweeps. As mentioned in an earlier paragraph, examination of the dead and dying larvae in the test plots showed that virtually all of the mortality was the result of infection by *B. thuringiensis*. Microscopic examination of the diseased specimens revealed the presence of large numbers of the bacillus in the tissues and body fluids of the caterpillars. The presence of *B. thuringiensis* was also confirmed by cultural tests.

TABLE 1. NUMBERS (AVERAGE OF TWO-SWEEP SAMPLES) OF LIVING LARVAE IN TEST (TREATED) PLOTS 1 THROUGH 6 (SERIES I) AND CONTROL (UNTREATED) PLOTS 1 THROUGH 3.

Date, 1950	Daily temperature		Total counts of living larvae*										
			Test (treated) plots							Control (untreated) plots			
	Max.	Min.	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	Avg.†	Plot 1	Plot 2	Plot 3	Avg.‡
8-1	108	63	55	31	31	44	38	63	44	37	34	53	41
8-3	109	65	34	19	11	20	17	21	20	70	83	84	79
8-4	103	72	24	18	8	4	6	6	11	84	90	84	86

* Each figure is an average of five two-sweep counts.
† Average of total test plots.
‡ Average of total control plots.

TABLE 2. NUMBER (AVERAGE OF TWO-SWEEP SAMPLES) OF LIVING LARVAE IN TEST (TREATED) PLOTS 7 THROUGH 9 (SERIES I) AND CONTROL (UNTREATED) PLOTS 7 THROUGH 9.

Date, 1950 (hrs. after application)	Daily temperature		Total counts of living larvae*						Average total counts	
			Plot 7		Plot 8		Plot 9		Plots 7, 8, 9	
	Max.	Min.	Test plot	Control plot	Test plot	Control plot	Test plot	Control plot	Test plot	Control plot
8-29 (0 hours)	107	62	47	53	87	72	120	90	85	71
8-30 (34 hours)	105	60	18	49	42	78	36	101	32	76
8-31 (58 hours)	106	62	12	45	21	83	30	90	21	76
9-1 (82 hours)	108	63	9	61	11	80	29	89	16	77

* Each figure is an average of five two-sweep counts.

So far as individual plots are concerned, the most rapid drop occurred in plot 6 in which the count dropped from 63 to six larvae per two sweeps. On the other hand, the least rapid decline took place in plot 1 in which the count was reduced only from 55 to 24 in the three-day period. The reasons for this difference in the two plots are not clear. The fact that the dosage or spore concentration in plot 1 was somewhat lower than in plot 5 may have had some effect, but on the basis of certain laboratory observations this hardly seems a sufficient explanation.

Figure 12 is a graphic representation of the average counts obtained in the second series of test plots and controls (plots 7 to 9). The two-sweep counts were made in the same manner as in the first series of plots, and covered a

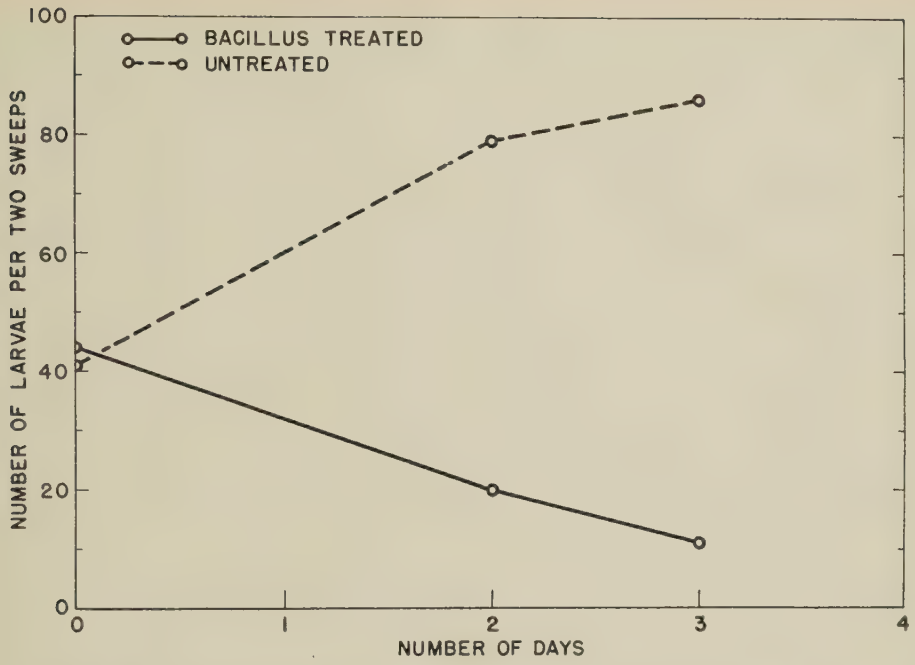

Fig. 11. The number of larvae per two sweeps in the test (treated) and control (untreated) plots. Average of test plots 1 through 6, and control plots 1 through 3. Based on data in table 1.

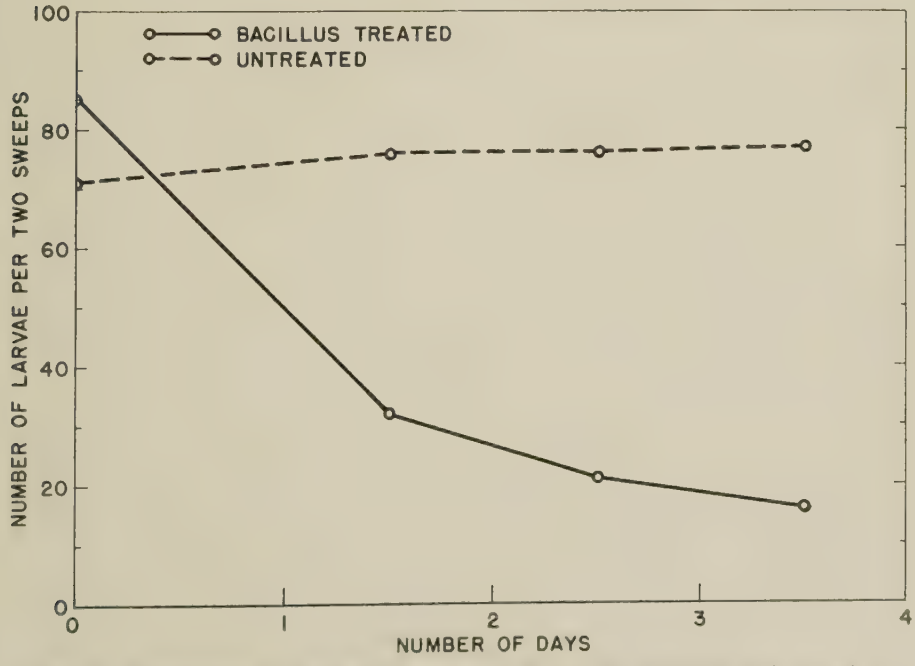

Fig. 12. The number of larvae per two sweeps in the test (treated) and control (untreated) plots. Average of test plots 7 through 9, and control plots 7 through 9. Based on data in table 2.

period of 82 hours—almost 3½ days. In this series the spore-spray was applied at a time when the alfalfa caterpillar population was almost at its maximum; consequently the population in the control plots did not continue to rise after the time of application as was the case in plots 1 to 6. Nevertheless, within 34 hours following the application of the spray the average count of plots 7, 8, and 9 was down to 32 per two sweeps from the initial count of 85. From this point until the cutting of the alfalfa necessitated the ending of the experiment 48 hours later, the population was reduced at a slower rate. The average count at the end of the experiment was 16 per two sweeps, having dropped to this point from an original high of 85 in 82 hours. Of the three plots in this series, the greatest drop occurred in plot 9 where the count was reduced from 120 to 36 in a 34-hour period, although not lowering a great deal after this point. Test plot 7, originally having a count of 47 larvae per two sweeps was reduced to only nine larvae in the 82-hour duration of the experiment. The population of all corresponding control (untreated) plots remained fairly constant (71 to 77 larvae per two sweeps) during the course of the experiment.

One of the most obvious population changes in this second series of test plots was the relatively rapid drop in numbers of larvae between the time of application and 34 hours later, with subsequent leveling off. Except possibly in plot 8, this was a rather consistent phenomenon in all the plots as indicated by the figures representing the average counts. The pattern is most apparent in the counts for plot 9. There is reason to believe that this leveling off of the curve would not have occurred to such an extent were it not for the fact that in these particular plots recent oviposition resulted in the hatching of large numbers of larvae at about the time the experiment was initiated. The greatest percentage of caterpillars in the last two sweepings (those made on August 31 and September 1) were small first, second, and third instar larvae, most of which had not as yet had the opportunity to develop the disease. Very few of the larger fourth and fifth instar larvae remained uninfected after 34 hours. It is conceivable, therefore, that had the experiment not been interrupted by the cutting of the alfalfa for hay, the caterpillar population which had been brought to below an economic level would have remained there as the result of the continuous infection of the new young larvae. Furthermore, since most of the population, as represented by the count of 16, were early instar larvae which consume very little food, the damage to the alfalfa crop under such circumstances would have been negligible.

Comparative Effectiveness of Bacillus and Virus

Comparing the results obtained in the foregoing experiments with those obtained during the past few years using a polyhedrosis virus (*Borrelina campeoles* Steinhaus) (Steinhaus, 1948; Steinhaus and Thompson, 1949; Thompson and Steinhaus, 1950; and Thompson, unpublished experiments) to control the alfalfa caterpillar, several interesting differences are apparent. In the first place it is clear, on the basis of the tests made so far, that although *B. thuringiensis* is capable of reducing a destructive population of the caterpillar to a point below which no economic damage to the crop occurs, it does

not as consistently suppress the population to as low a point as does the virus. Unfortunately, the cutting of the alfalfa for hay on the fourth day of the experiments did not permit continuing the tests for periods as long as those on which most of the virus data were based. Although in four of the nine test plots the bacillus did reduce the count to below 10 per two sweeps, perhaps greater reductions would have been recorded for the remainder of the plots had the tests been allowed to proceed unhindered. Nevertheless, on the basis of data so far obtained, the virus method does appear to be at least slightly more thorough in its effectiveness.

On the other hand there are at least two possible advantages of the bacillus over the virus, so far as controlling the alfalfa caterpillar may be concerned: the bacterium is more quickly effective, and the infection leaves the integument of the insect intact. Because of its longer incubation period the virus usually does not begin effectively to reduce the caterpillar population until five or six days after application. In the case of the bacillus, however, marked reduction in the number of insects occurred by the second day. In fact, greater reduction may take place during the first 48 hours than during any similar period thereafter.

When the alfalfa caterpillar is killed by the polyhedrosis virus, the integument usually disintegrates and breaks open, permitting the liquefied body contents to run out over the host plant. When this occurs on a large scale, the palatability of the hay for cattle is sometimes adversely affected. As far as could be determined in connection with the nine field tests using *B. thuringiensis,* when the caterpillar dies of infection with the bacillus, its integument ordinarily remains intact. Most of the dead larvae apparently drop to the ground without smearing the alfalfa plant.

Under some circumstances the ability of *B. thuringiensis* to grow on ordinary artificial media may be considered an advantage over the polyhedrosis virus, which is known to multiply only in the living tissue of its insect host. Because of this characteristic the bacillus may be simply and easily produced in large quantity with the resulting dry product being clean and easily stored. Actually, however, this is not as great an advantage over the virus as may first appear. Large quantities of the virus may be collected by sweeping up the diseased larvae with a net from infested fields, and storing under refrigeration which reduces the somewhat disagreeable odor that accompanies the virus preparations. Such a method eliminates the expense of the ingredients of the artificial media. With considerably more work and time than are now employed, it would be possible to prepare relatively pure preparations of virus-containing polyhedra that could be dried and stored in a manner similar to that described for the bacterial spores.

Because of the comparative speed with which the bacillus infects the alfalfa caterpillar and the relative thoroughness with which the virus is effective in suppressing the insect, it would appear that a combination of the two agents might constitute a more highly efficient means of control than the use of either one alone. Such a bivalent product would be simple to make up in the field just prior to the time of application. It could also be prepared previous to the time of application if the virus material as well as the spore material were in the form of a powder or dust. Following the application of the com-

bined materials one could expect to have an initial rapid drop (within 48 hours) in the caterpillar population as a result of infection by the bacillus, followed three or four days later by a further reduction in the remaining population as a result of infection by the virus. Whether such desirable results would actually occur in practice can only be ascertained experimentally. It is hoped that such an experiment can be completed during next season's work.

As they are now prepared, the cost of producing the bacterial spores is likely to be somewhat greater than that necessary to make field collections of polyhedrosis virus. The greater cost would be connected primarily with the cost of the media required to grow the bacillus and the greater amount of technical help required to maintain production. Of course, it is possible that adequate amounts of infectious material could be gathered from fields infested with bacterially diseased larvae in a manner similar to that now recommended for the virus. Unless it was desired to take the time necessary to free the bacterial suspensions of the dead insect tissue, the material could be stored in the refrigerator, as is done with the virus material, to suppress putrefaction and the accompanying odors. The practicability of such a procedure would depend on whether or not sufficient numbers of spores are produced in the cadavers of the diseased insects. Alfalfa caterpillars infected with *B. thuringiensis* usually contain large numbers of vegetative cells, but what percentage of these form resistant spores has not been determined. Although no actual figures are available on the cost of producing spore material, it would not appear to be great and is probably not greater than the cost of most chemical insecticides. The same apparatus is used to apply the spore-spray or dust as is used with chemical insecticides, and the cost of application of the two is about the same.

As is the case with the polyhedrosis virus, so with *B. thuringiensis* due caution must be exercised in considering the possible use of the pathogen as an economical and practical means of controlling the alfalfa caterpillar. Certainly no definite conclusion can be made as to the efficacy or practicability of the method until further experimentation with the bacillus has been accomplished in the laboratory and in the field. The mere fact that *B. thuringiensis* is markedly pathogenic for some insects under certain conditions and that it retains its virulence when cultivated repeatedly on artificial media or when stored in a dried condition makes it an inviting organism with which to work, especially when one considers its potentialities as a control agent not only against the alfalfa caterpillar but against certain other insects as well.

SUMMARY

The sporeforming bacterium *Bacillus thuringiensis* Berliner, known in the literature as pathogenic for the larvae of such insects as the Mediterranean flour moth and the European corn borer, has been found to be virulent for certain Californian insects, particularly the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval. Although taxonomically *B. thuringiensis* is a strain of *Bacillus cereus* Frankland and Frankland, the present specific name is retained because of the distinct pathogenicity of this strain for insects com-

pared to the lack of such pathogenicity with most other strains of *B. cereus*.

In addition to *B. thuringiensis* and *B. cereus*, 37 strains of various well-known species of sporeformers were tested for their capacity to infect the alfalfa caterpillar, the western yellow-striped armyworm, the California oakworm, the variegated cutworm, and the buckeye caterpillar. Only one of the 37 strains exhibited any significant degree of pathogenicity for the test larvae. This was a strain of *Bacillus thuringiensis* originally isolated from a diseased larva of the moth *Aphomia gularis* (Zeller).

Alfalfa caterpillars sick or dying of the disease show those symptoms of sluggishness, diarrhea, discoloration, and flaccidity typical of most bacterial infections. After death the body of the caterpillar becomes gradually more darkened, the body contents may be of a soft consistency, and there is present a more or less characteristic putrefying odor.

On the basis of the distinct virulence shown in the laboratory by *B. thuringiensis* for the alfalfa caterpillar, preliminary field tests were conducted to determine the susceptibility of the insect as it might occur under field conditions. Nine test plots near Dos Palos, California, infested with the caterpillar were sprayed with spore suspensions of the bacillus. Within 24 to 48 hours following such applications, infection of the insects was vigorous enough to kill large numbers of the caterpillar. In seven of the nine test plots the population was brought to below an economic level (20 larvae per two sweeps), whereas in each of the untreated control plots the population remained at a destructive level. In the remaining two test plots the count was brought down to just slightly above the economic level, and would very probably have been reduced still further in a day or two had the plots not been cut for hay.

Further tests and experimentation will have to be made before any recommendations can be given as to the possible use of *B. thuringiensis* in the control of the alfalfa caterpillar. Although the bacillus is capable of reducing a destructive population of the insect to a point below which no economic damage to the crop occurs, it does not appear to suppress the population as consistently to as low a point as does the polyhedrosis virus, which also affects this insect. On the other hand, the bacillus has several advantages over the virus, the two most important being the speed with which it is effective (about two days in the case of the bacillus; five or six days in the case of the virus), and the intactness with which the infection leaves the integument of the insect, thus allowing the dead insect to fall to the ground without smearing the alfalfa.

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